pivotal importance. Our group has shown that vesicular stomatitis virus G protein-

pseudotyped lentiviral vectors (LVs) delivered systemically to mice and non-human primates efficiently target and transduce liver cells, including liver resident macrophages termed Kupffer cells (KCs). Building on these findings, we developed a novel LV-based platform, termed KC-LV, to selectively engineer KCs in vivo with the goal of delivering therapeutic molecules specifically to LMS. To this aim, the KC-LV design exploits a reconstituted mannose receptor c type 1 (MRC1) promoter, active in macrophages, including KCs. To further fine-tune KC specificity, the KC-LV also includes microRNA target (miRT) sequences that prohibit LV transgene expression in liver sinusoidal endothelial cells and hepatocytes. We observed that systemic delivery to mice of a KC-LV driving the fluorescent marker GFP resulted in transgene product expression in KCs, but not in other liver cell types or organs, such as brain, gut, lung, lymph nodes and bone marrow. In mice bearing LMS, GFP expression was enriched in the tissue rim surrounding LMS. To leverage KCLV as a therapeutic platform we included a sequence encoding for interferon a (IFNα), a cytokine with pleotropic immune function. Long term analysis of mice treated systemically with IFNα KC-LV showed a rapidly established, vector dose-dependent and sustained IFNα expression, with no signs of hepatotoxicity, neutropenia or macroscopic skin reactions. IFNα KC-LV systemically delivered to mice challenged with colorectal cancer LMS, either obtained from cancer cell lines or organoids, significantly delayed tumor growth and achieved, in some mice, a complete response. Furthermore, mice that completely cleared LMS were refractory to rechallenge with matched cancer cells indicating persisting adaptive immune protection. Single cell RNA sequencing analysis of LMS revealed upregulation of IFN-responsive genes and altered activation/ polarization profile in tumor infiltrating host cells in mice treated with IFNα KC-LV indicating robust immune reprogramming of the LMS microenvironment. In particular, IFNα KC-LV promoted macrophage skewing from a protumoral (M2-like) to an antitumoral (M1-like) polarization state and expansion of LMS specific CD8 T cells. In summary, we have developed an innovative gene-based platform that upon a single well-tolerated intravenous LV infusion can rapidly establish a protective response against mouse LMS through promotion of macrophage reprograming and adaptive immune activation.

114. Inducible Tumor-Targeted Interferon-α Gene Therapy Inhibits Glioblastoma Multiforme in Mouse Model without Adverse Systemic Effects

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limit clinical application. The possibility to specifically target cytokines to the tumor establishing a local source that maintains physiologicalrange concentrations may thus open up new therapeutic perspectives. To this aim, our group has exploited a population of tumor-associated TIE2 expressing macrophages (TEM) to deliver IFN-α specifically to tumors through a cell and gene therapy approach. Transplantation of hematopoietic stem cells (HSCs) transduced with lentiviral vector (LV) expressing *Ifna1* under the control of the *Tie2* promoter induces TEMspecific release of the cytokine in the tumor microenvironment (TME), reprogramming it towards immunological activation.Glioblastoma multiforme (GBM) is the most common and aggressive brain tumor. The unsuccessful results of current therapies make GBM treatment an unmet clinical need. Its strongly immunosuppressive TME and the poor drug delivery across the blood brain barrier, make GBM a suitable candidate for testing our cell-based gene therapy. **Methods:** Mice were transplanted with HSCs transduced with *Tie2-Ifna1* LV. To further improve our platform, we developed an inducible strategy based on fusing destabilizing domains (DD) to the cytokine, thus allowing control of the timing and amount of IFN-α secreted in the TME. Both strategies were tested in a new syngeneic mouse model of GBM, called mGB2, which closely recapitulates several features of the human pathology. **Results:**Upon GBM challenge, mice transplanted with *Tie2-Ifna1* LV transduced HSCs showed remarkable tumor inhibition and improved survival over controls. Notably, a fraction of mice cleared the tumor and survived long term suggesting induction of protective immunity. Similar results were obtained with the inducible platform. The tumor-targeted loco-temporal regulated release of IFN-α induced anti-cancer effect in stringent therapeutic settings, when it was switched ON in already established tumors, and was associated with more effective, durable and safer profile compared to systemic treatment with recombinant IFN-α. Single-cell RNA sequencing and FACS analysis of the tumor infiltrate revealed substantial activation of IFN responsive genes in monocyte/macrophages, B cells and dendritic cells, accompanied by decreased exhaustion of T cells. Moreover, IFN-α gene therapy treatment polarized tumor-associated macrophages (TAMs) towards a pro-inflammatory phenotype, while impairing the recruitment and/or induction of an immunosuppressive subset of TAM, whose gene signature was found to have negative prognostic value in the human disease. **Conclusions:** These results indicate that IFN-α gene therapy can reprogram the GBM TME and stimulate anti-cancer immune response. The promising pre-clinical results obtained so far from our group led to the registration of the first drug based on this strategy, named Temferon®, which is currently in phase I/II clinical trial in our Institute for the treatment of patients with GBM. Our data also demonstrate the feasibility of inducible and intra-tumoral targeted delivery of an immune activating cytokine, thus opening the way to broader application to other cytokines and tumor types.

115. Design and Demonstration of Potent In Vitro and In Vivo Activity for CART-ddBCMA,

a BCMA-Targeted CAR-T Cell Therapy Incorporating a Non-scFv Binding Domain

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Chimeric Antigen Receptor (CAR) T cell therapies directed against B-cell maturation antigen (BCMA) have demonstrated compelling clinical activity and manageable safety in subjects with relapsed and refractory Multiple Myeloma (RRMM). Prior reported CAR-T cells have encoded humanized or murine scFvs, or camelid heavy chain antibody fragments with 41BB or CD28 co-stimulatory domains. Herein, we describe the generation and preclinical evaluation of CART-ddBCMA, an anti-BCMA CAR-T cell therapy encoding a non-scFv, synthetic binding domain targeting BCMA with a 4-1BB costimulatory motif and CD3-zeta T cell activation domain. The BCMA binding domain was discovered from a phage display library of D domains based on a ~8 kD highly stable scaffold and engineered for reduced immunogenicity. A human tissue cross-reactivity study using the BCMA binding domain as the test article demonstrated that the binding domain was specific for BCMA. Preclinical in vitro studies of CART-ddBCMA cocultured with BCMA-positive cell lines demonstrated highly potent, dose-dependent measures of cytotoxicity, cytokine production, T cell degranulation, and T cell proliferation. Furthermore, in each assay CART-ddBCMA performed as well as T cells incorporating the BCMA-directed scFv-based C11D5.3 CAR. CART-ddBCMA demonstrated in vivo efficacy in a disseminated BCMA-expressing tumor model in NSG™ immunocompromised mice. Mice engrafted with 5×10^6 U266 cells and grown for 37 days rapidly cleared tumor burden within 7 days following injection of 4.5×10^6, 1.5×10^6, or 0.5×10^6 CART-ddBCMA cells, and tumor clearance was sustained throughout the duration of the experiment. The level of CART-ddBCMA cells detected in circulation 14 days post-T cell transfer was dependent on the initial dose, and at each dose CAR expression was maintained in 90+% of hCD3+ T cells. Based on these promising preclinical data CART-ddBCMA is being studied in a firstin-human phase I clinical study to assess the safety, pharmacokinetics, immunogenicity, efficacy, and duration of effect for patients with RRMM (NCT04155749).

116. A SOX2 Engineered Epigenetic Silencer Factor Represses the Cancer Genetic Program and Eradicate Glioblastoma Development

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increase the overall survival. Patients usually undergo tumor resection followed by adjuvant radio- and chemo-therapies that can't prevent recurrences. It has been proposed that some stem cell-like cancer cells (CSCs) with tumor-initiating potential remain in preserved tissue, resist the adjuvant treatments, leading to the re-appearance of the disease. By rational engineering of the SOX2 cancer-associated transcription factor, we generated a synthetic epigenetic repressor named as SOX2 Epigenetic Silencer (SES) that maintains the ability to bind to a large group of its original targets inducing their long-term silencing. By deleting the C-terminal domain of SOX2 and fusing two epigenetic repressor domains, SES is able to effectively inhibit the SOX2 tumorigenic molecular network (rather than activating it as the original TF), including genes crucial for tumor maintenance and growth. By doing this, SES kills both glioma cell lines and patient-derived CSCs both *in vitro* and *in vivo*. Following *in situ*viral transduction of GBM xenografts in mice, we have demonstrated that SES induced a strong growth inhibition of pre-formed tumor of human origin. Remarkably, we have also proved that SES is not harmful to neurons and glia and does not trigger significant transcriptional changes in these cells. Nevertheless, we equipped the vector for our anti-cancer therapy with either a cell-cycle specific promoter or a micro-RNA binding sequence which restricts SES expression exclusively in cancer cells. These data suggest that in situ viral delivery of SES can target the residual cancer cells spread in the tissues and inhibit their growth and survival while leaving unaffected brain parenchyma cells. This approach enables the development of synthetic epigenetic silencers by engineering oncogenic transcription factors that can repress entire tumorigenic transcriptional pathways that are critical for supporting cancer development. Our strategy paves the way to an unprecedented therapeutic opportunity for the treatment of aggressive brain tumors endowed with high levels of efficacy and safety.

117. Oncolytic Adeno-Immunotherapy Expressing IL-12p70 and Immune Checkpoint Blockade PD-L1 Minibody Modulates the Host Immune System to Enable HER2.CAR T-Cells to Cure Pancreatic Tumors

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Oncolytic Adenoviral vectors (OAds) encoding immunomodulatory transgenes ("Armed"OAds) have shown promise as cancer immunotherapy agents. To overcome the limited transgene capacity of OAds, we have combined OAd with a Helper-dependent Ad (HDAd) (CAd system) to locally provide both oncolysis and multiple immunomodulatory molecules encoded by an HDAd. While intratumoral administration of CAd is insufficient to cure metastasized tumors, we have previously demonstrated that combination of local CAd injection with systemic human epidermal growth factor receptor 2 (HER2) specific chimeric antigen receptor T-cells (HER2. CART) provides significant anti-tumor activity in various solid tumor xenograft models including orthotopic metastatic

[1] San Raffaele Scientific Institute, Milano, Italy,[2]Humanitas University, Milano, Italy Glioblastoma multiforme (GBM) is the most common and lethal brain cancer in adults (1-5 cases per 100,000 people per year, 12-15 months of median survival). This is due, in addition to the aggressiveness of the disease, to the inefficacy of current therapies to substantially

model. Based on these encouraging preclinical studies, we proposed this combination immunotherapy with CAd producing IL-12p70, PD-L1

blocking antibody, and an HSVtk safety switch (CAd*Trio*) to treat patients with HER2 positive solid malignancies, including pancreatic adenocarcinoma (PDAC) because high expression levels of HER2 have been associated with poor prognosis in PDAC patients. Since treatment with single immunotherapies (checkpoint inhibitor, oncolytic virus, CAR T-cell) showed limited anti-tumor efficacy in PDAC patients, in this study we evaluated whether our combination immunotherapy enables control of PDAC growth in multiple PDAC models including humanized mice. Combinatorial treatment with CAd*Trio* and HER2. CART cures PDAC tumors within 25 days without tumor recurrence in xenograft models. HER2.CART is the dominant contributor of our combination therapy to the anti-tumor activity in xenograft PDAC models. However, in one tumor model, CAd*Trio* enhanced early HER2.CART infiltration/expansion at the tumor site, leading to better overall anti-tumor activity than treatment with HER2.CART alone. To address how PDAC tumor microenvironment contributes to our immunotherapy, we evaluated our immunotherapies in humanized mouse models. We found that intratumoral CAd*Trio* treatment induced type I IFN responses, including chemotaxis, to enable HER2. CART migration to the tumor site resulting in significant tumor control, and long-term complete responses. In contrast to xenograft models, HER2.CART treatment alone could not cure PDAC tumors in humanized mice. Additionally, using an advanced PDAC tumor model in humanized mice, we found that local CAd*Trio* treatment repolarized distant tumor microenvironments, leading to improved HER2.CART anti-tumor activity at a metastatic tumor site. Elucidation of immune gene signatures indicate CAd*Trio*-dependent stimulation of NK cells, together with *in vitro* data showing CAd*Trio* components and type I IFN induced by CAd*Trio* enhance NK cell anti-PDAC activity, suggest that host immune cells activated through our immunotherapy contribute to the overall anti-tumor effect of our combinatorial treatment. Our data demonstrate that CAd*Trio* and HER2.CART provide complementary activities to eliminate PDAC tumors and may represent a promising therapy for PDAC patients. We have initiated a Phase I clinical trial at BCM (NCT03740256) that has recruited its first patients to test CAd*Trio* with HER2.CART in patients with HER2positive solid tumors including PDAC.

118. UCARTCS1A, an Allogeneic CAR T-Cell Therapy Targeting CS1 in Patients with Relapsed/Refractory Multiple Myeloma (RRMM): Preliminary Translational Results from a First-in-Human Phase I Trial (MELANI-01)

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MELANI-01 (NCT04142619) is a phase I dose-escalation trial of UCARTCS1A, an allogeneic chimeric antigen receptor (CAR) T-cell therapy targeting the CD2 subset-1 (CS1) antigen in patients (pts) with RRMM. UCARTCS1A production includes knockout of the T-cell receptor (to minimize the risk of GVHD) and CS1 (to avoid fratricide during manufacturing). After lymphodepletion with fludarabine/cyclophosphamide, 5 pts received UCARTCS1A: 4 pts in dose level 1 (DL1) (1×10^6 cells/kg) and 1 pt in DL2 (3×10^6 cells/kg). Clinical response per IMWG criteria and exploratory correlates including CS1 expression on MM cells, UCARTCS1A expansion and persistence, changes in serum biomarkers (cytokines, ferritin, liver enzymes), and host immune cell reconstitution were assessed. In all pts, CS1 was expressed in >97% of detected plasma cells. After administration, UCARTCS1A cells were detected in 3/5 pts, with expansion observed in 1 DL1 pt and 1 DL2 pt. In the DL1 pt (4 prior lines of MM therapy), peak UCARTCS1A level in whole blood by flow cytometry was 78 cells/µL and vector copy number (VCN) was 27,385 copies/µg DNA at day (d) 28. This pt achieved an MRD-negative partial response (PR) at d28 and a very good PR (VGPR) by month (mo) 3. Serum lambda light chains (LC) decreased from 503.2 to <1.3 mg/L and M protein from 2.9 to 0.1 g/dL between d0 and mo 3, with LC detectable only by immunofixation at mo 3. Notable clinical findings included grade (G) 2 cytokine release syndrome (CRS), CAR-related hemophagocytic lymphohistiocytosis (HLH), and elevated serum biomarkers including IL-6, IL-8, and IFNγ. This pt died on d109 from organizing pneumonia in the context of prolonged severe lymphopenia in the absence of myeloma progression. In the DL2 pt (13 prior lines of MM therapy), peak UCARTCS1A level was 42 cells/µL (d11) and VCN was 141,090 copies/µg DNA on d9. Serum M protein and kappa LC decreased from 0.9 to 0.4 g/dL and from 15.4 to 1.4 mg/L, respectively, from d0 to d14. The pt achieved a PR at d14. Notable clinical findings included G4 CRS. This pt died on d25 as a result of G5 hemorrhagic pancreatitis in the context of CRS, CAR-related HLH, disseminated mucormycosis, and pseudomonal pneumonia. Among the 3 nonresponding pts in DL1 (11 to 15 prior lines of MM therapy), 1 pt maintained stable disease and 2 pts experienced disease progression. UCARTCS1A cell expansion was associated with meaningful clinical responses, though with notable toxicity including G2-G4 CRS and G5 events. After a brief clinical hold, the study has resumed with protocol updates to address the potential risk of prolonged lymphopenia and severe CRS. These preliminary results support further investigation of UCARTCS1A in RRMM.

119. A Drug-Regulated Anti-CD33 Chimeric

Antigen Receptor with Potent Anti-AML Activity and a Reversible On-Off Switch

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fused to the rapamycin (RAPA)-dependent dimerization domains FRB* and FKBP12. RAPA heterodimerizes antigen binding and signaling receptors, reconstituting CD33-dependent T cell activation. Here, we probe pharmacologic control of DARIC33 T cells using systems for pulsatile RAPA dosing. We find that DARIC33 T cells show nearly complete RAPA-dependent anti-CD33 activity and in vivo efficacy comparable to a clinically validated CD19 CAR. **Results:** DARIC33 T cells preactivated with RAPA for 24hr were washed and cultured in RAPA-free media for different periods prior to stimulation with CD33+ target cells in vitro. Pre-activated DARIC33 T cells showed high levels of cytokine release followed by a progressive decline to baseline levels. Re-addition of RAPA restored antigendependent cytokine release to levels of DARIC33 T cells maintained in RAPA continuously. We also found DARIC33 T cells efficiently eliminate CD33+ spheroids in the presence of RAPA. Following spheroid challenge, DARIC33 T cells were isolated and cultured in RAPA-free media for 48 hours, followed by rechallenge with a second spheroid target. DARIC33 T cells exhibited significantly reduced cytotoxicity after RAPA wash out. However, re-addition of RAPA rescued T cell cytotoxicity, leading to complete spheroid clearance. Thus two in vitro systems show DARIC33 T cell activation by RAPA and reduced cytokine release or cytotoxicity following RAPA withdrawal. In both systems, depressed effector function of DARIC33 T cells following RAPA withdrawal can be restored through re-addition of RAPA. To assess RAPA modulation of DARIC33 activity in vivo, NSG mice engrafted with CD33+ MV4-11 AML cells were treated with DARIC33 T cells + various durations of RAPA. Mice treated continuously with RAPA cleared tumors, whereas mice treated for only 1 week showed tumor regression followed by relapse, confirming RAPA withdrawal deactivates DARIC33 T cells. Next, Raji lymphoma cells, engineered to express CD33 at levels similar to CD19, were injected into NSG mice. The engrafted CD33+CD19+ Raji tumors were then treated with DARIC33 T cells or control CD19 CAR T cells in the presence or absence of RAPA. Mice treated with 10e6 CD19 CAR T cells or 30e6 DARIC33 T cells + RAPA controlled Raji tumor growth. Anti-tumor activity was not observed in mice treated with 30e6 DARIC33 T cells in the absence of RAPA. **Conclusions:** These data demonstrate that DARIC33 T cells exert potent anti-AML activity that can be modulated with RAPA. The resulting temporal control of T cell activation may enable

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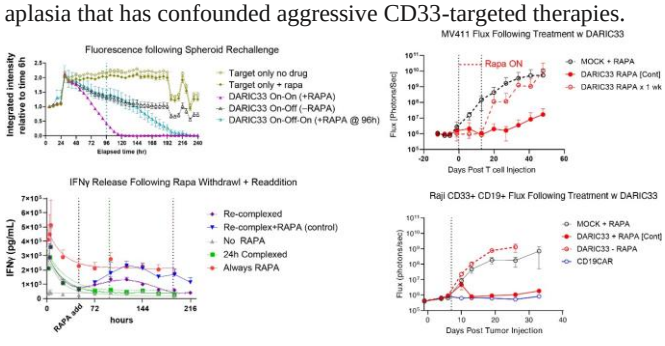
Background: Acute myeloid leukemia (AML) antigens are expressed on normal hematopoietic precursors. Regulated CAR T cells are an attractive strategy for achieving (1) therapeutic efficacy, (2) hematopoietic recovery, and (3) exhaustion avoidance by temporally constraining antigen-dependent activation. We previously described dimerizing agent regulated immunoreceptor complex targeting CD33 (DARIC33), wherein antigen binding and T cell signaling domains of a chimeric antigen receptor are spatially separated and

optimization of durable effector function, and avoid long-term aplasia that has confounded aggressive CD33-targeted therapies.



Upstream Process Development for AAV Vector Production

120. Co-Identification and Characterization of Host and Viral Protein Interactomes during AAV Production by Two Different Proximity Labeling Methods

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Scalable manufacturing of high titer and high potency vectors is a challenge for translating recombinant Adeno-Associated Virus (rAAV)-based gene therapeutics into clinical applications. Efficiencies in AAV genome replication, capsid assembly and genome encapsidation are the keys to the success of vector production. Understanding the interactomes in producer cells during AAV vector production will provide insights into developing more efficient production platforms. The proximity-dependent biotin identification method allows detecting protein-protein interactions with a promiscuous biotin ligase fused to a bait protein. These biotinylated proteins can be selectively isolated by biotin-streptavidin capture and identified by mass spectrometry. We grafted two types of biotin ligase, BioID2 or TurboID onto the surface of AAV2 capsid. BioID2 is a smaller biotin ligase and the major disadvantage is its slow kinetics, which necessitates labeling with biotin for 18-24 hours. By contrast, TurboID is greater in size but enable the labeling in ten minutes. Both engineered AAV2 capsids can biotinylate un-biotinylated substrates just like a purified biotin ligase. During AAV vector production by using 293 cell transient transfection method, biotin and ATP were added to the culture medium. Seventytwo hours post transfection, proteins were then selectively isolated by streptavidin-coated beads for mass spectrometry analysis. Rep proteins were found biotinylated regardless of the presence of packageable genomes, suggesting the formation of Rep-Capsid complex before genome encapsidation.

This pre-encapsidation complexes were also previously detected by coimmunoprecipitation, co-sedimentation, and yeast two-hybrid analyses (Ralf Dubielzig, et al., 1999). In addition, a total of 61 cellular proteins were co-identified by both BioID2 and TurboID technologies as direct or indirect interactors with viral proteins possibly involved in vector genome replication, capsid assembly, and genome encapsidation. The main predicted biological functions of the 61 cellular coding genes are associated with cell cycle, immune response, and cell growth by GO term analysis. Ongoing studies aim to further validate contributions of those cellular genes to AAV production process. Our findings may shed some lights to developing strategies to improve vector empty/full particle ratios and virus genome titer. *Co-corresponding authors.

121. The Effects of ITR Structure and Plasmid Backbone on Plasmid Stability and Yield

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In viral vector production, plasmid integrity is of paramount importance. Plasmid yield is the number one KPI in plasmid

manufacturing, affecting process design, product quality and overall cost. It is common knowledge that AAV ITR-carrying plasmids tend to be unstable, resulting in plasmid variants, extra DNA fragments, and low production yield. Here we present a comprehensive study to document these changes and to understand the causes of ITR instability by means of NGS and restriction analyses. We found that both ITR structure and plasmid backbone contributed to such instability. Our data showed that modification of either ITR structure or plasmid backbone would reduce, and even eliminate, deletions in ITR and result in an increase of plasmid yield from 30% to several folds.

122. Increasing Gene Therapy Vector Production Using Viral Sensitizer Molecules

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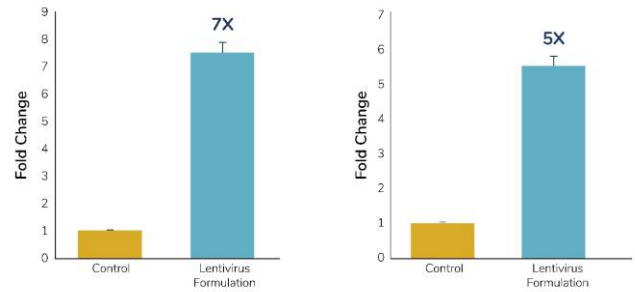
Inefficient manufacturing processes can have several important implications during product development. The first and most obvious is cost of goods (COGs), which is driven upwards when GMP compliant material is required. Related are the practical considerations of manufacturing virus using an inefficient process, which means longer times to produce target amounts of virus, more substantial needs in personnel, physical space, and investments in infrastructure. Lastly, there is the issue of maximum feasible dose, where the maximum dose of a manufactured virus given to a patient is limited by the amount of virus that can be produced. Viral Sensitizers (VSETMs) encompass a proprietary collection of small molecules that enhance the growth of viruses by transiently and efficiently overcoming cellular antiviral defenses. VSEs can be used in a range of applications such as improving virus manufacturing yield, improving tumor infection by oncolytic viruses, or transduction of cells by common gene therapy vectors like AAV, adenovirus, and lentivirus. Owing to different molecular mechanisms through which they operate, VSEs can be combined, adapted, and formulated for specific uses. To improve lentivirus production, we employed high throughput screening and DOE methodology to develop a VSE formulation that could improve 3rd generation lentivirus production in adherent HEK293T cells (VSE-LentiTM). We designed a highthroughput method to identify single then multi-VSE compound formulations that enhance lentivirus production. Different transfection reagents, and production scales as well as commonly used lentivirus production enhancers were tested. We found VSE-LentiTM to be compatible with several transfection reagents (PEI Pro, lipofectamine, TransIT lenti). VSE-LentiTM was able to enhance lentivirus production whether added at the time of transfection or up to 24h prior to transfection. Enhancements in lentivirus production were observed over 72 hours, without a requirement to resupply compounds. Improvements in yield observed using VSE-LentiTM were condition-dependent but in pre-optimized conditions lentivirus production conditions, VSE-LentiTM led to >5X increase in TU/ml. **Figure 1. VSE-Lenti™ molecules were added to adherent HEK293T cells at the time of transfection in combination with PEI transfection reagent. Following transfection with luciferase-expressing 3rd generation lentivirus plasmids, a 5-7-fold increase in luciferase activity was observed.)**



123. Transcriptional Response of HEK293 cells to Clinical-Scale Recombinant Adeno Associated Virus Production by Transient Transfection

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As a leading viral vector for *in vivo* gene therapy, AAV is nonpathogenic and can effectively infect a wide range of human tissues. Currently, most AAV-based gene therapy pipelines rely on AAV manufacturing via transient triple plasmids transfection in human HEK293 cells. However, the productivity of the platform

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The Pfizer gene therapy platform relies on transient transfection of HEK293 cells with three plasmids encoding the viral genes required for rAAV replication and assembly. Improving rAAV production is hampered by a lack of fundamental understanding of host cell cofactors and cellular responses to AAV production. We hypothesized that rAAV productivity is constrained by cellular stress or antiviral responses. To investigate potential bottlenecks to productivity, we defined the kinetic transcriptional response of HEK293 cells to rAAV production in a clinical manufacturing-relevant process. We found ~2000 genes differentially expressed after transient transfection and during rAAV production compared with pre-transfection control. Pathways involved in detection of external stimulus, defense response to virus and inflammatory response were regulated at different times post transfection. This indicates that multiplexed cellular responses were triggered by rAAV production, which may negatively impact rAAV productivity. Systematic analyses of the cellular transcriptional response to rAAV production may illuminate genes limiting rAAV yields, thereby enabling the rational design of a next-generation rAAV manufacturing platforms.

124. Vector Engineering of pRep-Cap and pHelper Enhanced AAV Productivity by Triple Transfection in Suspension HEK293 Cells

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AAV for clinical development and commercialization. Previously, we isolated a highly productive clonal cell 5B8 in suspension HEK293, and established a robust cGMP AAV manufacturing platform that was based on triple transfection and supported production in single-use bioreactor up to 250L scale for multiple AAV serotypes. To continue to improve our platform, we designed and evaluated a panel of pRepCap and pHelper plasmid vectors for AAV production. For pRep-Cap vector, we focused on rebalancing expression of Rep and Cap. Through engineering of their native viral promoters, we identified the best combination of Rep-Cap expression levels in pLHI_Rep-Cap [a] that could double the AAV titer across multiple serotypes, e.g. AAV2, AAV5, AAV9, and Anc80L65. The improvement was confirmed with different AAV transgenes in multiple runs in 250L scale. For pHelper vector, we screened five additional candidate helper genes and/or isoforms. Remarkably, we found that the additional co-transfection of one candidate, but not others, with standard triple plasmids could increase the AAV titer 50%-100% over just standard triple transfection method. Consistently, the enhancement was dose-dependent, as more plasmids co-transfection resulted in higher AAV titer up to 100% more. Furthermore, we subcloned the overexpression cassette into the current state-of-the-art vector to create a new generation of pHelper plasmid, named pLHI_Helper [b]. The pLHI_Helper plasmid could be manufactured at the same cost as regular pHelpler but would provide more than 50% productivity compared to the state-of-the-art vector. Taken together, our study suggested that optimization of vector design in AAV manufacturing plasmids is feasible and can significantly improve the AAV productivity in large scale by triple transfection in suspension HEK293 cells. [a, b] Patent pending.

125. High Titer rAAV Production upon Upstream Process Development of Stable Helper-Virus Free ELEVECTA® Producer Cells

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In recent years, the number of gene therapy products in the biopharma clinical pipeline have increased, as well as the need for scalable manufacturing processes for viral vector production. Recombinant adeno-associated virus (rAAV) is widely used as viral gene therapy vector, however the delivery of the required amounts of AAV-vector particles is still a challenge. We have recently developed a stable helpervirus free rAAV production platform named ELEVECTA®. This AAV production platform consists of mammalian suspension cells which have stably integrated all components necessary to produce AAV, namely the adenovirus helper functions E2A, E4ORF6, VA RNA, as well as AAV replicase, AAV capsid and the gene of interest (GOI) flanked with the AAV ITRs. Production in this system is initiated by induction via doxycycline. Stable rAAV production using the ELEVECTA platform has been proven with different serotypes as well as different GOIs. To further develop the production process using the ELEVECTA® production platform, upstream process optimization was performed with one of CEVEC's AAV8 proof of concept single cell clones. The optimized upstream process, developed in the ambr® 15 was first scaled to 10 L, then in a collaboration with Pall Corporation it was successfully scaled-up to 200 L using the Allegro™ single-use stirred tank bioreactor. A major advantage of fully stable AAV producer cell lines is that this allows for thorough upstream process development. In order to deploy an intensified large-scale process for rAAV production, we applied ATF perfusion technology. The ATF-based perfusion set-up at lab-scale consisted of a stirred tank bioreactor connected to an ATF unit. The cells were inoculated in chemically defined medium free of animal-derived components and cultivated in standard conditions. After reaching the target viable cell density, rAAV production was induced. The harvest was done from the whole cell suspension 4 to 5 days post-induction. Noteworthy, this ATF perfusion process resulted in very high titers (E15 vg/L) and high percentage of full particles (35-40 %).

needs to be improved in order to meet the growing demand of recombinant

126. Genome-Wide CRISPR Activation Screen Reveals That SKA2 and ITPRIP Increase AAV Manufacturing via Cell Cycle Modulation

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Introduction and Methods Recombinant adeno-associated viruses (rAAV) are a leading gene delivery platform; however, current rAAV manufacturing methods cannot meet the demands of the field, which will increasingly be a barrier for the clinical development and commercialization of gene therapies. To address this issue, we developed an AAV-specific genome-wide screening strategy to identify gene targets whose upregulation promotes rAAV packaging. Specifically, an AAV vector library carrying Synergistic Activation Mediator (SAM) guide RNA libraries was generated, and HEK293T cells expressing SAM machinery (a dCas9-VP64 fusion with MS2P65-HSF1) were infected with the AAV library for 48 hours to provide time for modulation of target host factor expression. The cells were then transfected with rAAV packaging genes (pAAV2) and pHelper for another 72 hours to package the rAAV with each vector genome. After the iterative rounds of selection, Next Generation Sequencing was used to analyze the AAV genomes and thus the guide RNAs targeting host factors that increased rAAV packaging. **Results** The most enriched guide RNA targeted the spindle and kinetochore associated complex subunit 2 (SKA2) transcript variant 2 and inositol 1, 4, 5-trisphosphate receptor interacting protein (ITPRIP). In the SKA2 or ITPRIP expressing stable cell line, AAV packaging was increased by 2.2-fold and 3.3-fold, respectively. A cell line expressing both SKA2 and ITPRIP increased AAV titer by 3.8-fold. We analyzed multiple mechanistic steps where target gene expression could impact AAV vector production, including transfection, viral gene expression, the cell cycle, and others. We found that AAV vector genome replication in SKA2 or ITPRIP expressing cell line was higher than that in WT cells. In addition, during AAV production ITPRIP expression increases the proportion of cells in the S-phase, when AAV vector genome replication is known to occur. Moreover, EdU incorporation in SKA2 expressing cells showed elevated S-phase synthesis, suggesting SKA2 may influence cell cycle kinetics during rAAV packaging. Consistent with this finding, culturing in high confluency to arrest in the G0 phase antagonized the effect of SKA2 and ITPRIP, indicating the effect of SKA2 and ITPRIP in rAAV packaging is

mediated by cell cycle modulation. Finally, we performed capsid ELISA and immunoblotting using crude lysate samples normalized by same copy number or same protein concentration and showed expression of SKA2 and ITPRIP increased the AAV full/empty capsid ratio, which suggests increased vector genome replication promoted genome loading into virions. **Conclusions** This broad screening strategy offers a new approach to improve rAAV producing cell lines as well as to unveil the host factors relating to virus packaging. Taken together, our genome wide activation screens revealed host factors, SKA2 and ITPRIP, that increased vector genome replication, the full capsid ratio, and consequently AAV production. **Disclosure** C.B., D.S.O., and D.V.S. are inventors on patents related to cell lines for increased production of AAV. D.V.S is a co-founder of 4D Molecular Therapeutics, which develops novel rAAV therapeutic vectors for clinical use.

Presidential Symposium and Top Abstracts

127. Base Editing Rescues Sickle Cell Disease in Human Hematopoietic Stem Cells and in Mice

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Everette[2,3], Yoonjeong Jang[3], Christopher J. Podracky[3], Elizabeth Thaman[6], Christopher Lechauve[1], Akshay Sharma[7], Jordana M. Henderson[8], Michelle F. Richter[2,3],
Kevin T. Zhao[2,3], Shannon M. Miller[2,3], Tina Wang[2,3], Luke W. Koblan[2,3], Anton P. McCaffrey[8], John F. Tisdale[9], Theodosia A. Kalfa[6,10], Shondra M. PruettMiller[5], Shengdar Q. Tsai[1], Mitchell J. Weiss[1], David R.
Liu[2,3]

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Memphis, TN,[5]Cell and Molecular Biology, St. Jude Children's Research Hospital,
Memphis, TN,[6]Division of Hematology, Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, OH,[7]Bone Marrow Transplantation & Cellular Therapy, St. Jude Children's Research Hospital, Memphis, TN,[8]TriLink BioTechnologies, San Diego, CA,[9]Molecular and Clinical Hematology Branch, NHLBI/NIDDK/NIH, Bethesda, MD,[10]Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH

Sickle cell disease (SCD) is a catastrophic disorder caused by a mutation in the *HBB* gene encoding the β-globin subunit of adult hemoglobin. We used a bespoke adenine base editor (ABE8e-NRCH) to convert the SCD allele (HBB^S) to hemoglobin Makassar (HBB^G), a naturally occurring non-pathogenic allele. Delivery of mRNA encoding ABE8eNRCH and an HBB^S-targeting single guide RNA (sgRNA) into $CD34^+$

hematopoietic stem and progenitor cells (HSPCs) from patients with SCD using a clinical electroporation method resulted in 80% conversion of *HBB*^{S} to *HBB*^{G} with no perturbation of erythropoiesis. Reticulocytes from edited CD34$^+$ cells showed an 80% shift of total β-like globin protein from sickle (β^S) to Makassar (β^G), and a 3-fold decrease in hypoxia-induced sickling. Sixteen weeks after transfer of edited human CD34$^+$ cells into adult immunodeficient mice, the *HBB*^{S}-to-*HBB*^{G} editing frequency in engrafted cells was 68%, indicating durable modification of HSCs. Reticulocytes from the bone marrow of engrafted mice showed a shift of 63% of total β-like protein from β^S to β^G, and a 5-fold decrease in hypoxia-induced sickling. Since human red blood cells (RBCs) do not survive in mouse peripheral blood, we also examined the physiological effects of *HBB*^{S} base editing by electroporating ABE8e-NRCH and sgRNA ribonucleoprotein into HSPCs from a mouse harboring human SCD alleles, followed by transplantation into irradiated host mice. Sixteen weeks after transplantation, 56% of *HBB*^{S} alleles were converted to *HBB*^{G} in bone marrow-repopulating donor HSPCs. Circulating RBCs showed a shift of 84% of total β-like protein from β^S to β^G, and a 3-fold decrease in hypoxia-induced sickling. Mice that received base-edited HSPCs showed rescue of hematologic parameters to near-normal levels and reduced splenic pathology compared to mice transplanted with unmodified HSPCs. Secondary transplantation of edited bone marrow confirmed durable phenotypic rescue of long-term hematopoietic stem cells and when performed with different ratios of edited and unedited HSPCs revealed that ≥20% *HBB*^{S}-to-*HBB*^{G} editing is required for phenotypic rescue, confirming that base editing treatment substantially exceeds this threshold. Human HSPCs showed evidence of p53 activation and large DNA deletions or rearrangements following treatment with Cas9 nuclease targeting *BCL11A*, but not following treatment with ABE8e-NRCH targeting *HBB*^{S}. These findings suggest a potential one-time autologous treatment for individuals with SCD that eliminates pathogenic *HBB*^{S}, generates benign *HBB*^{G}, and minimizes undesired consequences of making double-strand DNA breaks.

128. Safety and Efficacy Results with a Single Dose of Autologous CRISPR-Cas9-Modified CD34$^+$ Hematopoietic Stem and Progenitor Cells (HSPCs) in Transfusion-Dependent β-Thalassemia (TDT) and Sickle Cell Disease (SCD)

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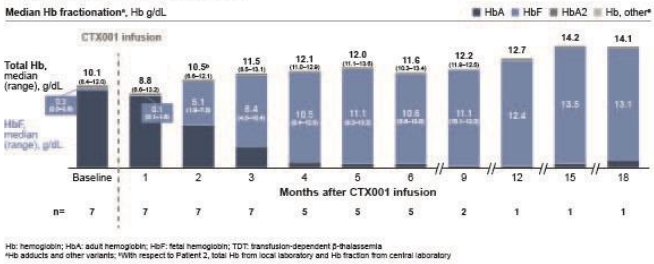
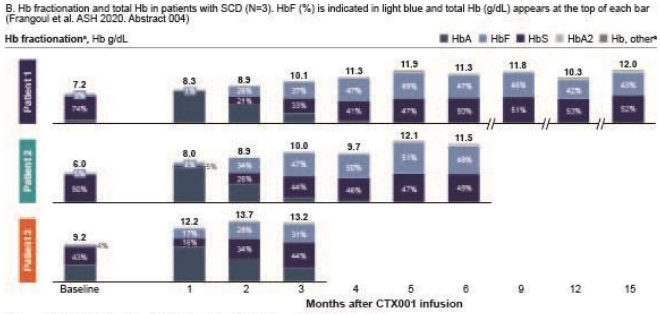
BCL11A is a key transcription factor that suppresses the production of fetal hemoglobin (HbF) in red blood cells (RBCs). In TDT and SCD, elevated HbF is associated with fewer transfusions and clinical complications. To reactivate HbF in RBCs we used the CRISPR-Cas9 platform to edit the erythroid enhancer region of *BCL11A* in HSPCs ex vivo (CTX001™). We present safety and efficacy results from patients (pts) infused with CTX001 with ≥3 months (mo) of follow-up (f/u). CLIMB THAL-111 (TDT, NCT03655678) and CLIMB SCD-121 (SCD, NCT03745287) are multicenter, first-in-human studies of CTX001. Pts aged 12-35 years (ys) with TDT (all genotypes) receiving ≥10 units/y of packed RBC transfusions in the prior 2 ys, and those with severe SCD (≥2 vaso-occlusive crises (VOCs)/y requiring medical care in the prior 2 ys) were eligible. We collected peripheral CD34$^+$ HSPCs by apheresis after mobilization with G-CSF and plerixafor (TDT) or plerixafor alone (SCD). We edited the erythroid enhancer region of *BCL11A* in the enriched CD34$^+$ cells using a specific CRISPR guide-RNA and Cas9 nuclease. Pts received myeloablative busulfan before infusion. We monitored engraftment, AEs, total Hb, HbF, hemolysis, F-cells, RBC transfusions (TDT), and VOCs (SCD). 7 TDT pts (median f/u 8.9 mo, range 3.8-21.5) and 3 SCD pts (median f/u 7.8 mo, 3.8-16.6) received CTX001. Median neutrophil and platelet engraftment were achieved on Day 32 (20-39) and 37 (29-52) respectively in TDT pts, and on Day 22 (17-30) and 30 (30-33) in SCD pts. The safety profile after infusion was generally consistent with myeloablative conditioning and autologous bone marrow transplant. 4 serious AEs (SAEs) related or possibly related to CTX001 occurred in 1 TDT pt in the context of HLH: HLH, headache, ARDS, and IPS. All resolved at time of analysis. The other 9 pts reported no CTX001-related SAEs. Pts received their last transfusion within 2 mo after infusion and showed increases in Hb and HbF over time (Figure). The first pts have been transfusion-free for >20.5 (TDT) and >16.0 (SCD) mo. No SCD pts have had a VOC since infusion. The first SCD pt has been VOC-free for >16.6 mo. CTX001 led to increases in HbF and total Hb in all treated pts. Its post-infusion safety profile is generally consistent with myeloablation. All 7 TDT pts have been transfusion-free for ~2 mo and the 3 SCD pts have had no VOCs. These early data demonstrate CTX001 is a potential functional cure for treatment of TDT and SCD.

A. Median Hb fractionation and total Hb in patients with TDT (N=7). HbF (g/dL) is indicated in light blue and total Hb (g/dL) appears at the top of each bar (Frangoul et al. ASH 2020. Abstract 004)



Hb: hemoglobin; HbA: adult hemoglobin; HbF: fetal hemoglobin; TDT: transfusion-dependent β-thalassemia
*Hb adducts and other variants; †With respect to Patient 2, total Hb from local laboratory and Hb fraction from central laboratory

B. Hb fractionation and total Hb in patients with SCD (N=3). HbF (%) is indicated in light blue and total Hb (g/dL) appears at the top of each bar (Frangoul et al. ASH 2020. Abstract 004)



Hb: hemoglobin; HbA: adult hemoglobin; HbF: fetal hemoglobin; HbS: sickle hemoglobin; SCD: sickle cell disease
*Hb adducts and other variants

129. Immunostimulatory Bacterial Antigen-Armed Oncolytic Measles Virotherapy Significantly Increases the Potency of Anti-PD1 Checkpoint Therapy

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was CD8$^+$T cell dependent. Inhibition of the IFN response pathway using the JAK1/JAK2 inhibitor ruxolitinib significantly decreased PDL1 expression on myeloid-derived suppressor cells (MDSCs) in the brain and potentiated the therapeutic effect of MV-s-NAP-uPA and anti-PD1. Our findings support that measles virus strains armed with bacterial immunostimulatory antigens represent an effective strategy to overcome the limited efficacy of immune checkpoint inhibitor based therapies in GBM creating a novel and promising translational strategy for this lethal brain tumor.

130. In-Vivo Engineered B Cells Retain Memory and Secrete High Titers of Anti-HIV Antibodies in Mice

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Eliciting a potent and neutralizing antibody response to diverse and rapidly mutating viruses is a long-standing clinical challenge. HIV specific broadly neutralizing antibodies (bNAbs) can suppress viremia, as demonstrated recently by combination therapy. However, the mean elimination half-life of the bNAbs in patients is shorter than a month, requiring constant administration to prevent the virus from rebounding. Moreover, individuals with prior HIV resistance to the antibodies are excluded from trials and resistance to one antibody occurs when the concentration of the second diminishes. Persistence may be addressed by constitutive expression, however anti-drug antibodies may develop, possibly due to improper glycosylation. B cell engineering provides an opportunity to express a bNAb for adaptive immunity. Both mucosal protection and systemic clearance may be achieved by Class Switch Recombination (CSR). Somatic Hypermutation (SHM) followed by affinity maturation may allow for counteracting viral escape and memory retention allows for increased titers upon viral resurgence. We recently demonstrated that, upon adoptive transfer, bNAb engineered B cells undergo differentiation, memory retention, CSR, SHM and clonal expansion. However, extensive, and expensive *ex-vivo* manipulations hinder clinical potential of this approach. Furthermore, allogeneic B cell therapy necessitates MHC-II compatibility to receive T cell help. To overcome these limitations, we engineer B cells *in-vivo*. In particular, we demonstrate that an injection of two AAV-DJ vectors, one coding for CRISPR/Cas9 and another coding for a bNAb donor cassette, allows for site specific integration in B cells. Following immunizations, we show memory retention and bNAb secretion at high titers. Antibodies secreted by the engineered B cells were found to be of multiple isotypes and IgGs could neutralize autologous and heterologous pseudoviruses. The engineered antibody coding genes underwent somatic hypermutation and clonal selection. Detected engineered cells by flow cytometry included B cells in the blood, plasmablasts and germinal center B cells in the spleen, indicating B lineage differentiation. Biodistribution of the donor AAV over time

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Clinical immunotherapy approaches are lacking efficacy in the treatment of glioblastoma (GBM). In this study, we sought to reverse local and systemic GBM-induced immunosuppression using the Helicobacter pylori neutrophil-activating protein (NAP), a potent TLR-2 agonist as a novel immunostimulatory transgene expressed in an oncolytic measles virus (MV) platform, retargeted to allow viral entry through the urokinase-type plasminogen activator receptor (uPAR). While single agent murine anti-PD1 treatment or repeat *in situ* immunization with MV-s-NAP-uPA provided modest survival benefit in MV resistant models, the combination treatment led to synergy with a cure rate of 80% in mice bearing intracranial GL261 ($P= 0.047$)and 72% in mice with CT-2A tumors ($P= 0.007$). Combination NAP-immunovirotherapy induced massive influx of lymphoid cells in mouse brain, with CD8$^+$ T cell predominance; therapeutic efficacy

indicated CRISPR-dependent expansion of engineered B cells only in lymphatic tissues. We further assessed the

possible off target effects of our *in vivo* B-cell engineering approach and found limited CRISPR/Cas9 off-target cleavage, using unbiased, highly sensitive, CHANGE-Seq analysis. Finally, we reduced on-target cleavage at undesired tissues by expressing Cas9 from a B cell specific promoter and by coding the gRNA in the donor vector. In summary, we demonstrate that B cells can be safely engineered *in-vivo*. We propose that *in-vivo* B cell engineering should be considered for novel future applications, to address other persistent infections or to treat autoimmune diseases, genetic disorders, and cancer.

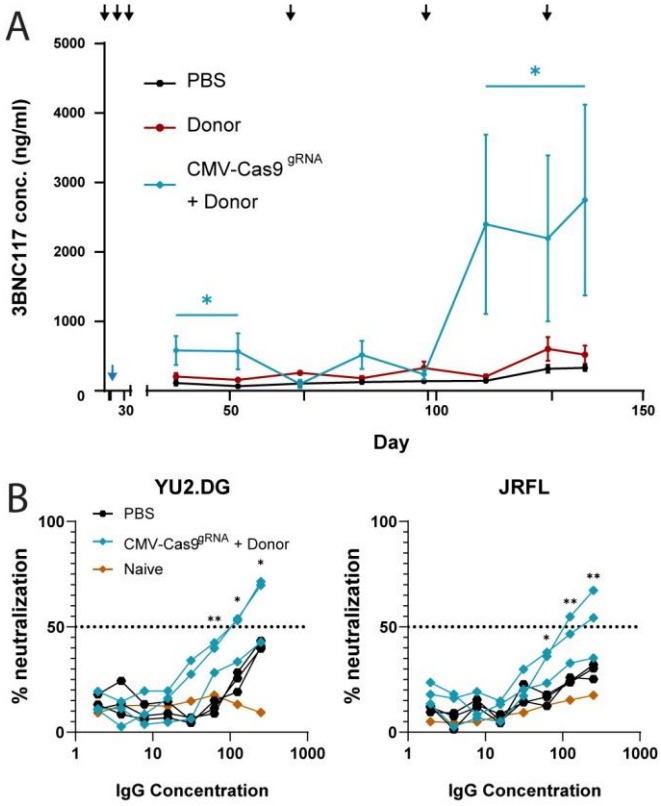

Figure: in-vivo engineered B cells retain memory and secrete high titers of anti-HIV antibodies in mice. A. Serum titers of the antibody integrated in the cells (3BNC117). Black arrows indicate immunizations, blue arrow indicate AAV injection. B. Engineered B cells secrete functional bNAbs. Neutralization of autologous (YU2.DG, left) and heterologous (JRFL, right) pseudoviruses using purified IgGs from last time point as in A.

AAV Therapies for Neurological and Sensory Diseases

131. AAV-Mediated GJB2 Gene Therapy Rescues Hearing Loss and Cochlear Damage in a Mouse Model of Congenital Hearing Loss Caused by Conditional Connexin26 Knockout

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According to the NIDCD, 2-3 out of every 1,000 children in the United States are born with some degree of hearing loss, with more than half due to genetic factors. Mutations in the *GJB2* gene which encodes the gap junction protein Connexin 26 (CX26) are the most common forms of non-syndromic deafness, responsible for approx. 50% of cases. While in most subjects the onset of hearing loss is prelingual and moderate to severe, in some subjects, hearing loss due to loss of CX26 is mild and progressive. In the inner ear, expression of CX26 is vital for the function of various non-sensory cell types including support cells and fibrocytes. Results from mouse and human studies have revealed that mutations in *Cx26* ultimately lead to near total degeneration of cochlear hair cells. Since the constitutive homozygous *Cx26* knockout is embryonic lethal, we utilized conditional knockouts to study the effect of losing CX26 protein in the cells of the inner ear. We utilized two different conditional knockout strains (*Cx26* cKO) generated by crossing $Cx26^{loxp/loxp}$ mice with either an inducible *cre* mouse line or with a constitutive *cre* mouse line. Using the inducible *cre* line, we knocked out *Cx26* with temporal control and observed varying degrees of hearing loss and cochlear defects dependent on the time of *cre* induction. Early postnatal *cre* induction caused severe to profound hearing loss in the *Cx26* cKO mice when assessed at postnatal day 30, whereas later induction of *cre* resulted in mild to moderate hearing loss that was progressive in nature. Constitutive *cre Cx26* cKO animals, by virtue of embryonic *cre* expression in the inner ear tissues, displayed severe to profound hearing loss across all frequencies tested. The availability of these various mouse models enabled us to evaluate AAV-mediated *GJB2* gene therapy across a spectrum of hearing loss severity that mimics known human phenotypes. We previously reported the identification of novel adeno-associated viral (AAV) vectors that produce efficient expression of a gene of interest in cochlear support cells in rodents and non-human primates. We designed an AAV vector with an optimized capsid, promoter and human *GJB2* gene elements (OTO-825) that provides excellent expression of CX26 in cochlear support cells and fibrocytes. We also generated an identical AAV vector that expresses CX26 with a FLAG-tag to allow identification of virally expressed CX26 (OTO-825-FLAG). In cell-based assays, utilizing HeLa cells that do not normally express CX26, both OTO-825 and OTO-825-FLAG induced expression of CX26 that was correctly trafficked to the cell membrane. Injection of OTO-825-FLAG into the cochleae of mice provided near total expression of CX26-FLAG in our cells of interest throughout the cochlea (from base to apex). Compared with vehicle, intracochlear administration of OTO-825 to postnatal *Cx26* cKO mice substantially restored CX26 expression and provided a marked improvement in hearing across multiple frequencies. In addition, OTO-825-injected *Cx26* cKO mice had greatly improved cochlear morphology relative to those injected with